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Review

Methods for detection of point mutations: performance and quality assessment

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We give an overview of current methods for the detection of point mutations as well as small insertions and deletions in clinical diagnostics. For each method, the following characteristics are specified: (*a*) principle, (*b*) major modifications, (*c*) maximum fragment size that can be analyzed, (*d*) ratio and type of mutations that can be detected, (*e*) minimum ratio of mutant to wild-type alleles at which mutations can be detected, and (*f*) detection methods. Special attention is paid to the possibilities of quality assessment and the potential for standardization and automation.

INDEXING TERMS: alleles • electrophoresis • gene insertions • gene deletions • polymerase chain reaction

A variety of methods for the detection of point mutations as well as small deletions or insertions has been described. For the appropriate choice of any one of these methods, several criteria must be considered:

1) What type of nucleic acid is analyzed (DNA or RNA)?

2) What kind of specimen is analyzed (e.g., peripheral blood, bone marrow, tissues, secretions, excretions)?

3) Are the mutations to be detected known before analysis?

4) How large is the number of potential mutations to be detected?

5) Need each of the potential mutations be detected?

6) What is the ratio between wild-type and mutant alleles?

7) How reliable is the method to be used, and how far can it be standardized?

8) How does the test perform?

9) Is the test suited for routine diagnosis?

10) What kind of quality assessment can be achieved?

Here, different methods for the detection of point mutations and small deletions or insertions will be discussed on the basis of the above criteria (for simplification, we shall refer to point mutations only in the text, though in general, small deletions or insertions are detected equally well by the methods described). In general, PCR is either used for the generation of DNA fragments, or is part of the detection method. Screening methods for unknown mutations as well as methods for the detection of known mutations are included. Though DNA sequencing techniques will not be covered, we stress that DNA sequencing is considered the gold standard and remains the definitive procedure for the detection of mutations so far. For this reason, mutations assumed from the results of screening methods must be confirmed by DNA sequencing. Special attention will be paid to performance and quality assessment. We do not intend to present an in-depth review. For detailed information the reader is referred to some review articles *[1, 2]*.

Screening Methods

Disregarding direct sequencing of PCR products, two different approaches for the detection of unknown point mutations can be distinguished. One set of methods relies on the differences in electrophoretic mobilities of wild-type and mutant nucleic acids. The second group of methods is based on the cleavage of heteroduplices. Recently, a new principle that depends on the association of mismatch binding proteins with mismatches in heteroduplices has been described.

In general, target sequences are amplified by PCR before analysis. At present, *Taq* polymerase is widely used for amplification. The error rate of *Taq* polymerase is in the range of 10^{-4} to 10^{-5} per nucleotide and is strongly affected by the reaction conditions (e.g., concentrations of magnesium chloride and dNTPs, pH, and temperature). Depending on the method of choice, polymerase errors may contribute reasonably to unspecific background, limiting the level of detection, particularly in situations where few mutated alleles are analyzed in a great excess

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of wild-type alleles (for theoretical considerations see ref. 3). Though at low statistical probability, errors may be misinterpreted as mutations when analyses are performed with low numbers of starting templates (<100 molecules; *[4]*). If polymerase errors are critical, positive results should be confirmed by alternative techniques and, though not applicable to all methods, thermostable polymerases with higher fidelity (e.g., *Pfu* DNA polymerase) may improve results in particular applications.

DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE), TEMPERATURE GRADIENT GEL ELECTROPHORESIS (TGGE)[1]

Principle. Double-stranded (ds) DNA is electrophoresed through a gradient of increasing concentration of a denaturing agent (urea or formamide) or of increasing temperature. With increasing concentration of denaturant or temperature, domains in the DNA dissociate according to their melting temperature (T_m). DNA hybrids of 100–1000 bp contain 2–5 such domains, each melting at a distinct temperature. Dissociation of strands in such domains results in a decrease in electrophoretic mobility. A 1-bp difference between two ds DNA homoduplices can change the T_m by 1 °C or more. Base mismatches in heteroduplices lead to a significant destabilization of domains, resulting in differences of T_m between homoduplex and heteroduplex of up to 6 °C. For this reason, heteroduplices between wild-type and mutant fragments are generally used for the analysis of point mutations. Theoretical melting profiles can be predicted by appropriate computer programs *[5]* (for a detailed review on DGGE see ref. 6).

Modifications. To increase the number of melting domains to be analyzed, GC-rich sequences are attached to one of the PCR primers (GC clamp). With GC clamps, significantly more mutations were detected by DGGE *[7, 8]*.

Fragment size. Maximum fragment size suited for DGGE is ~1000 bp. With increasing number of melting domains, the mobility shifts decrease. For this reason, the fraction of mutations detected decreases with increasing fragment size. In addition, time of separation varies from 7.5 h to 10 h for fragment sizes in the range of 50 to 1000 bp.

Detectable mutations. According to data from the literature and our own experiences, close to 100% of point mutations can be detected when heteroduplices are generated from sense and antisense strands and when GC clamps are attached *[6, 8, 9]*.

Detection limit. DGGE or TGGE appears not to be suited for the detection of a few mutant alleles in great excess of wild-type alleles, since preselection of mutant alleles is not feasible.

Detection methods. In the original report, radioactive labeling of DNA fragments was performed *[10]*. Radioactive labeling has been replaced by ethidium bromide or silver stain.

Performance and quality assessment. Before analysis, optimal conditions for DGGE or TGGE must be determined either by calculation on the basis of appropriate algorithms or by experimental perpendicular gradient gel electrophoresis. In general, optimal separation is evaluated experimentally. For both DGGE and TGGE, special equipment is commercially available.

In principle, four bands are detectable in a heterozygous state after denaturation and renaturation corresponding to two homodimers (WW', MM') and two heterodimers (WM', W'M). With homozygous germline mutations, four bands are detectable only after the addition of wild-type DNA. The relative intensities of bands depend on the quantitative relation of mutant to wild-type DNA. This can pose difficulties, especially in solid tumors with variable amounts of nontumor DNA.

SINGLE-STRAND CONFORMATION POLYMORPHISM (SSCP)

Principle. Under certain conditions, single-stranded (ss) nucleic acids form secondary structures in solution. The secondary structure depends on the base composition and may be altered by a single nucleotide exchange, causing differences in electrophoretic mobility under nondenaturing conditions *[11]*.

Modifications. Initially, SSCP was described for the analysis of DNA; however, analysis of RNA is also possible *[12, 13]*. Distinct secondary structures are formed more frequently by RNA than by DNA molecules. In comparison with DNA-SSCP, an additional step of in vitro transcription is required to generate RNA from PCR fragments *[13]*. With RNA, larger fragments can be analyzed *[13]*. Screening of multiple fragments can be achieved by either restriction digest of larger PCR fragments *[14]* or multiplex PCR *[15, 16]*. To identify potential mutations, SSCP has been combined with direct DNA sequencing *[17]*. In several applications, minigels have been used instead of standard sequencing gels *[18, 19]*. However, whether the resolution in a small gel is as high as in sequencing gels has not been established. In other previous studies, mobility differences have been analyzed by capillary electrophoresis instead of gel electrophoresis *[20]*.

[1] Nonstandard abbreviations: DGGE, TGGE, denaturing, temperature gradient gel electrophoresis; ds, double stranded; ss, single stranded; SSCP, single-strand conformation polymorphism; HET, heteroduplex analysis; CCM, chemical cleavage method; EMC, enzyme mismatch cleavage; CFLP, cleavase fragment length polymorphism; PTT, protein truncation test; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; RT, reverse transcription; ASO, allele-specific oligonucleotide; RFLP, restriction fragment length polymorphism; PODGE, profiling of oligonucleotide dissociation gel electrophoresis; ASA, allele-specific amplification; ARMS, amplification refractory mutation system; ASPCR, allele-specific PCR; and OLA, oligonucleotide ligation assay

Fragment size. For optimal results, fragment size should be in the range of 150 to 200 bp *[21]*. The number of detectable mutations decreases when larger fragments are analyzed. For larger fragments, acceptable sensitivities may be achieved by RNA-SSCP *[13]*.

Detectable mutations. Under optimal conditions (fragment size <200 bp), ~80–90% of potential base exchanges are detectable by SSCP *[21]*. In addition to the size of the fragment, assay performance is dependent on the concentration of glycerol within the gel and the constancy of temperature during gel electrophoresis. Except for G to T transversions, there appears to be no significant effect of the type of base exchange on sensitivity *[21]*. In comparison with DNA-SSCP, higher sensitivities, especially for analysis of fragments of >200 bp, have been reported for RNA-SSCP *[13]*.

Limit of detection. Approximately one mutant cell is detectable in the presence of 10 normal cells *[22]*.

Methods of detection. Initially, fragments were labeled radioactively and detected by autoradiography. Nonradioactive detection, e.g., by silver staining, is feasible.

Performance and quality assessment. Electrophoretic separation is carried out in nondenaturing polyacrylamide slab gels. Depending on the polyacrylamide concentration, the size of the fragment, and the presence of glycerol within the gel, time of separation varies between 3 and 6 h. For higher resolution, special gel matrices are commercially available *[23]*. Heating of gels during gel electrophoresis must be avoided. Adequate convection of air is obligatory. Otherwise, no specialized equipment is needed. Composition of reagents (e.g., concentration of glycerol) and conditions of electrophoresis (e.g., concentration of acrylamide or time of electrophoresis) are dependent on the characteristics of the DNA fragments to be analyzed. Analysis of one fragment under different conditions may increase the rate of detectable mutations. Optimal conditions are largely determined empirically. Thus, standardization is limited.

When SSCP is analyzed by gel electrophoresis, differences in mobility are evaluated by visual inspection. Standardization is limited in this setting. Similarly, automation is difficult to achieve. With the use of capillary electrophoresis instead of gel electrophoresis, both standardization and automation may be improved and the turnaround time will be decreased.

HETERODUPLEX ANALYSIS (HET)

Principle. Heteroduplices are generated by heat denaturation and reannealing of a mixture of wild-type and mutant DNA molecules. In nondenaturing polyacrylamide gels, homoduplices and heteroduplices exhibit distinct electrophoretic mobilities.

Modifications. For higher resolution, special gel matrices (MDE) can be applied instead of polyacrylamide gels *[23]*. Sharpening of bands may be obtained by the separation of duplices in the presence of 15% urea. When ratios of mutant to wild-type alleles are undefined, wild-type DNA has to be added to the sample, allowing sufficient formation of heteroduplices for the detection of mutations *[24]*.

Size of fragment. The optimal fragment length for the detection of point mutations varies between 200 and 600 bp; the detection of mutations in PCR fragments of up to 900 bp has been reported *[25, 26]*.

Detectable mutations. Though the method is widely used for screening purposes, relatively few systematic studies on the fraction of mutations detectable have been published. The detection of mutations is mainly dependent on the position of the mismatch within the DNA fragment and the type of mismatch. White et al. *[27]* described the detection of eight of nine different mutations by application of heteroduplex analysis. In one report, all *p53* mutations investigated by SSCP were likewise detectable by heteroduplex analysis *[23]*. The proportion of point mutations detected by HET has been estimated to ~80% *[1]*.

Limit of detection. Systematic studies are lacking. The detection limit depends both on the relative signal intensity and the separation of heteroduplex vs homoduplex. Ratios of mutant to wild-type DNA of <1:5 may not be detectable (own observations).

Detection method. Homo- and heteroduplices are detected either by ethidium bromide or silver staining after gel electrophoresis.

Performance and quality assessment. Electrophoretic separation is carried out in nondenaturing polyacrylamide sequencing gels. Depending on fragment size, time for electrophoretic separation varies between 14 and 30 h. So far, the method is not suitable for automation but may be performed automatically in the future with drastically reduced times of electrophoretic separation by application of capillary electrophoresis *[28]*.

RNASE A CLEAVAGE METHOD

Principle. Under defined conditions, mismatches within RNA:RNA or RNA:DNA heteroduplices are cleaved by RNase A. After cleavage, labeled fragments are analyzed by denaturing gel electrophoresis.

Detectable mutations. Mutations of purine bases are cleaved with low efficiency or remain uncleaved. For this reason, by analysis of RNA:DNA heteroduplices, only 30% to 40% of the possible mutations are detectable.

When both DNA sense and antisense strands are analyzed, detection rate can be increased up to 70% *[29]*.

Limit of detection. No detailed information is available with respect to the maximum ratio of mutant to wild-type alleles at which the detection of mutant alleles is still possible.

Fragment size. The maximum size of RNA that can be analyzed is ~1000 bp. Analysis of larger fragments results in high background due to unspecific cleavage at sites of perfect base pairing. Incomplete separation of RNA:DNA duplices may occur when large fragments are separated under denaturing conditions, making interpretation of results difficult *[6]*.

Comment. Since only 70% of all types of mutations are detectable, the method appears not to be suited for screening purposes when compared with other methods. For this reason, aspects of routine application and quality assessment will not be discussed in detail.

CHEMICAL CLEAVAGE METHOD (CCM)

Principle. Mispaired nucleotides within heteroduplices are modified by chemical agents by using Maxam–Gilbert sequencing chemistry. Hydroxylamine reacts with mispaired cytosine residues, osmium tetroxide with mispaired thymine residues. DNA:DNA or DNA:RNA heteroduplices are cleaved by piperidine at the sites of chemical modification. If sense and antisense strands are analyzed, all point mutations will be detected. Unspecific cleavage of homoduplices does not present a problem when the method is performed appropriately *[30]*.

Modifications. Originally, the method was described for the analysis of DNA:DNA heteroduplices, but it may also be applied for the analysis of DNA:RNA heteroduplices *[1]*. When low amounts of mutant alleles are analyzed in a large background of wild-type DNA, sensitivity can be increased by separation and detection of fluorescence-labeled fragments on a DNA sequencer *[31]*.

Detectable mutations. In principle, all possible mutations are detectable by CCM *[1, 30]*. It has been reported that certain T:G mismatches are not modified by osmium tetroxide. However, when both sense and antisense strands are analyzed, a reliable detection of all types of point mutations is achieved.

Detection limit. By application of fluorescence labeling, down to one mutant cell was detectable in a background of 10 nonmutated cells when separation and detection of fragments was performed on a DNA sequencer apparatus *[31]*.

Fragment length. As outlined by Cotton *[1]*, DNA fragments of up to 2 kb can be analyzed by CCM.

Methods of detection. Cleaved fragments are analyzed by gel electrophoresis. Originally, ^{32}P-end-labeled fragments were used. Improved resolution of signals is obtained by labeling with ^{35}S *[32]*. In addition, silver staining may be applied for detection *[33]*. Fluorescence labeling was mentioned above *[31]*.

Performance and quality assessment. The major disadvantage of the CCM is the use of toxic substances. Because several steps of the reaction must be carried out under a fume hood, the potential for automation is limited. The major advantages of the CCM is the fact that all point mutations are detected when sense and antisense strands are analyzed. Furthermore, an objective measurement of reaction products is feasible. Thus, separation and detection of DNA fragments may be standardized by the application of fluorescence-labeled primers in conjunction with a DNA sequencer *[31]* or possibly by capillary electrophoresis in the future.

ENZYME MISMATCH CLEAVAGE (EMC)

Principle. Heteroduplices generated by heat denaturation and renaturation of PCR products of polymorphic DNA or wild-type and mutant alleles, respectively, are incubated and cleaved by either the bacteriophage T4 endonuclease VII or T7 endonuclease I (bacteriophage resolvases). Subsequently, DNA fragments are analyzed by gel electrophoresis *[34, 35]*.

Fragment size. Mutations were detectable in PCR products between 88 and 940 bp *[34]* or up to 1.5 kb *[35]*.

Detectable mutations. By application of both enzymes in parallel, cleavage of heteroduplices was observed with all types of small deletions (1- and 3-bp deletions in the *APC* gene or the *CFTR* gene, respectively) and 13 of 14 point mutations representing all types of possible nucleotide exchanges. By application of only one enzyme, 11 of 14 mutations were identified. Although G to A transitions were detectable in most cases analyzed, the G to A exchange of the G551D mutation in the *CFTR* gene remained undetectable even when both enzymes were applied *[34]*. In a second report, 17 of 18 point mutations and 3 of 4 small deletions were detectable by application of T4 endonuclease VII only *[35]*. Both reports observed nonspecific background bands of undetermined origin.

Limit of detection. So far, bacteriophage resolvases have been applied to the analysis of heterozygous states. No systematic studies regarding the least ratio of mutant to wild-type alleles detectable are known to us.

Method of detection. In both reports mentioned above *[34, 35]*, ^{32}P-labeled primers were used for PCR. Subsequently, products were incubated with resolvases, separated on polyacrylamide gels under nondenaturing or

denaturing conditions, and fragments detected by autoradiography. Silver staining should be feasible.

Performance and quality assessment. A number of problems have to be solved before EMC can be considered as a routine screening method for mutations. With the enzyme preparations used so far, unspecific cleavage of homoduplices has been observed. The use of highly purified enzymes may solve this problem. Since homozygous mutant samples should not generate a specific signal, wild-type DNA has to be added to detect these mutations. Some mutations are poorly recognized by resolvases, resulting in digestion of only a small fraction of the DNA. It has been suggested that mutant resolvases may be developed that tightly bind a mismatch, but fail to cut it. This would allow the detection of mutations in a solid-phase assay *[36]*. Since unspecific cleavage of homoduplices may occur, homoduplices must be included as internal negative controls. The occurrence of unspecific bands may pose a problem for the correct interpretation of results. Additional experience with the use of resolvases is required before detailed suggestions on quality assessment can be given.

CLEAVASE FRAGMENT LENGTH POLYMORPHISM (CFLP)

Principle. CFLP analysis is a relatively recent method *[37]*. When single strands of DNA refold after denaturation, sequence-dependent secondary structures consisting of folded, hairpin-like configurations are formed. The cleavase I endonuclease cleaves just 5′ of the hairpin loop at the junction between ss and ds DNA, generating a collection of fragments that is unique to that strand of DNA. Changes in the sequence (e.g., single point mutations, insertions, or deletions) of that strand will alter the secondary structures formed and the CFLP pattern detected.

Fragment size. Fragments of up to 2 kb can be analyzed.

Detectable mutations. No systematic studies are available yet.

Limit of detection. No systematic studies are available yet.

Method of detection. CFLP patterns are resolved on short, denaturing polyacrylamide gels. Detection of fragments proceeds through labeling (e.g., biotin, ^{32}P) of one of the PCR primers.

Performance and quality assessment. Automation may be feasible by use of fluorescein-labeled primers in conjunction with capillary electrophoresis. As a control, wild-type alleles must be run in parallel. Additional experience is needed for detailed suggestions regarding quality assessment.

MUTATION DETECTION BY MISMATCH BINDING PROTEINS

Principle. Mutations are detected by binding of the MutS protein, a component of the *Escherichia coli* DNA mismatch repair system, to ds DNA molecules containing mismatched bases *[38]*.

Modifications. Heteroduplices were generated by heat denaturation and subsequent reannealing after PCR amplification of wild-type and mutant alleles. DNA duplices were incubated with the MutS protein, and mutations were detected by mobility shift assays *[38]*. A simple assay is based on the fact that MutS binding protects heteroduplex DNA from exonuclease digestion *[39]*. It has been suggested that a solid-phase assay may be feasible, in which immobilized MutS binds mismatches in heteroduplices.

Detectable mutations. MutS binds different mismatches with different affinities, and some mismatches (e.g., A:C) are bound poorly *[40]*. According to Lishansky et al. *[38]*, MutS bound more strongly to *CFTR* gene heteroduplices containing the ΔF_{508} 3-bp deletion in one of the strands than to heteroduplices with a single base-pair mismatch. The G542X and G551D point mutations of the *CFTR* gene were successfully detectable by the MutS assays *[38]*. In the MutS protection assay, three G-A and a C-T exchange were detected *[39]*. Recently, a solid-phase assay version with immobilized MutS has been described *[41]*.

Limit of detection. Until now, only heterozygous situations have been investigated. Systematic analysis of the detection of mutations in a high background of wild-type DNA are lacking.

Methods of detection. In the report by Lishansky et al. *[38]*, mutations were detected by gel mobility shift assays with ^{32}P-labeled PCR products. In the MutS protection assay, fluorescence-labeled fragments were detected after separation on a polyacrylamide sequencing gel *[39]*.

Performance and quality assessment. Reliability of the assay depends primarily on the discrimination of MutS binding to heteroduplices against the background binding to homoduplices. Unspecific signals have been reported for homoduplices consisting of either wild-type alleles or ΔF_{508} alleles. However, differences in signal intensities between homo- and heteroduplices allowed the correct interpretation *[38]*. In the same report, a lower stability of the DNA–protein complex and decreased signal-to-noise ratios have been described for point mutations in comparison with the ΔF_{508} deletion in the *CFTR* gene. Unspecific background binding has been speculated to result from errors of the *Taq* polymerase. However, because unspecific binding of MutS to homoduplices did not increase with fragment length and because the use of a polymerase of higher fidelity (*Pfu* polymerase) did not

reduce unspecific binding, this assumption has not yet been verified [38].

Recently, the human mismatch binding factor has been shown to consist of two proteins, the MutS homolog hMSH2 and a second protein, GTBP [42]. Since both proteins are required for mismatch binding, in vitro detection of mismatches may be improved by the use of both proteins. It has been suggested that artificial mismatch binding proteins may be generated from resolvases by site-directed mutagenesis. These mutant resolvases would tightly bind a mismatch, but fail to cut it [36]. Possibly, the use of different mismatch binding proteins may allow the detection of mutations in a simple solid-phase assay format in the future.

So far, several problems must be solved before the detection of mismatches in heteroduplices by binding proteins can be applied as a screening method for point mutations. For this reason, considerations regarding standardization and quality assessment appear premature.

PROTEIN TRUNCATION TEST (PTT)

Principle. The PTT is based on a combination of PCR, transcription, and translation. The test selectively detects translation-terminating mutations, which are revealed on the protein level by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE). A T7 promoter and an eukaryotic translation initiation sequence is linked to a PCR primer [43, 44]. Subsequently, PCR products are used as templates in coupled transcription–translation reactions. The size of translation products is analyzed by gel electrophoresis. Stop codons generated by point or frameshift mutations lead to a premature stop of translation and to a reduced size of the translated proteins.

Modifications. The methodological approach may start with reverse transcription of mRNA. The resulting cDNA is amplified by PCR (RT-PCR). Gross rearrangements and mutations affecting splicing are detectable already by analysis of the RT-PCR products [43, 44]. Alternatively, mutational hotspot regions may be amplified from genomic DNA [44, 45]. The T7 promoter and an eukaryotic translation initiation sequence may be linked to a primer used for only one round of PCR amplification [44, 45] or to nested primers in a second round of amplification [43]. Chain-terminating mutations have also been detected by cloning of DNA fragments in-frame with a colorimetric marker gene (*lacZ*) followed by screening for the level of functional activity of the marker polypeptide (β-galactosidase) [46].

Size of fragments. Sequences as long as 4–5 kb generate enough PCR product to allow their subsequent transcription and translation [43]. The upper size limit seems to depend on the ability to generate sufficient full-size RT-PCR product and on the difference between the size of wild-type and truncated proteins.

Detectable mutations. The assay detects translation-terminating mutations generated by either point or frameshift mutations. Missense mutations are not detected. Polymorphisms or silent mutations do not pose a problem in this assay. The method is particularly well-suited for genes in which translation-terminating mutations dominate, e.g., for the analysis of the *APC* and *BRCA1* tumor suppressor genes in which >80% of mutations result in truncated proteins [44, 47] or the *NF1* (neurofibromatosis 1) gene in which PTT detected close to 70% of the mutations [48].

Detection limit. No report is known to us in which the minimum ratio of mutant to wild-type alleles has been analyzed.

Detection methods. In vitro translation has been performed in the presence of radioactively labeled amino acids. After electrophoresis, protein fragments were detected by autoradiography. In the cloning assay in which β-galactosidase was used as a marker, recombinant colonies were screened with blue/white color selection.

Performance and quality assessment. No special equipment beyond the standard equipment of a molecular genetic laboratory is needed.

Translation-terminating mutations close to the 5′ primer binding site are expected to result in very short translation products that might escape detection. Alternatively, if the truncating mutation is located near the binding site of the 3′ primer, the lengths of the truncated and the wild-type translation products might be so close that the two peptides cannot be resolved by SDS-PAGE analysis [45]. Additional bands, probably representing isoforms, e.g., due to alternative splicing or technical artifacts, may complicate the interpretation of results. So far, differences in electrophoretic mobility of truncated vs nontruncated proteins have been analyzed by visual inspection. In the future, proteins may be separated by chromatography or capillary electrophoresis, allowing an objective measurement of truncated proteins. Since PTT involves a number of steps (RT-PCR, in-vitro transcription and translation, gel electrophoresis), internal positive controls should be included.

ALLELE-SPECIFIC OLIGONUCLEOTIDE (ASO) HYBRIDIZATION ON DNA CHIPS

Principle. In DNA chips, the principle of ASO hybridization has been extended to a screening method for mutations (for recent reviews see refs. 49 and 50). This has been made possible by the fabrication of high-density oligonucleotide arrays. Oligonucleotides of known sequences are immobilized on appropriate surfaces. Given a consensus sequence, a set of four probes can be defined for each nucleotide in the target. Thus, to screen 1000 nucleotides for a mutation or polymorphism would require 4000 probes. Labeled target sequences are hybridized to the immobilized oligonucleotides. Because of their high reso-

lution, fluorescent dyes are best suited. In a commercial system (Affymetrix, Santa Clara, CA), hybridization of the targets to the array is detected by epifluorescence confocal scanning microscopy.

Modifications. As solid supports, surface-modified glass, polypropylene, or glass with small patches of polyacrylamide have been described *[50].* Arrays of oligonucleotides representing the complements of a known sequence are synthesized by using combinatorial methods. In the Affymetrix system, photolithographic methods are used in conjunction with nucleotide precursors with a photocleavable protecting group *[51, 52].* In the first report on the application for screening polymorphisms of the HIV-1 clade B protease (*pr*) gene, HIV-1 DNA or RNA was converted to a ds DNA amplicon containing T3 and T7 RNA promoter sequences. The PCR amplicon was transcribed with T7 or T3 RNA polymerase in the presence of fluorescein-labeled rUTP. Fluorescein-labeled RNA was fragmented by heating, hybridized to the chip, and analyzed *[53].*

Size of fragments. The size of the DNA or RNA fragment to be tested depends on number, size, and sequence of probes on the array. In the first application published, a 382-bp contiguous region of the HIV genome (*pr* gene) was analyzed by a high-density array of 1.28 × 1.28 cm glass surface consisting of 12.224 different oligonucleotide probes *[53].*

Detectable mutations. The *pr* chip mentioned above was capable of determining 98.1% and 99%, respectively, of the sense and antisense strands of four HIV-1 strains. When the sequences from both strands were analyzed, all of the 382 bases were correctly determined *[53].* In high-density oligonucleotide arrays, multiple mutations occurring proximal to one another can lead to noncalls or ambiguous calls.

Detection limit. So far, no information is available regarding the minimum ratio of mutant to wild-type alleles detectable by high-density oligonucleotide arrays.

Detection methods. For high-density arrays, fluorescence has major advantages over other labeling procedures. Multiple colors can be used to label different sequences, resolution is high, and real-time measurements can be done. Both kinetic and equilibrium data can be collected.

Performance and quality assessment. For each target sequence, a special chip containing appropriate oligonucleotides has to be fabricated. Furthermore, a GeneChip-Scanner (Affymetrix) detection instrument is needed. Because of limited experience, no definite suggestions regarding quality assessment can be given at present.

Screening Methods for Point Mutations and Small Deletions or Insertions: Summary and Conclusions

Screening methods for point mutations and small deletions or insertions suited for diagnostic applications are summarized in Table 1.

SSCP and HET are the most simple among the screening tests. A major advantage of HET over other methods is that running conditions do not have to be optimized, as conditions are constant for the majority of fragments and time for optimal separation of different sized fragments can be predicted. For SSCP, screening should be performed under different running conditions to achieve a maximum sensitivity, making this method more time consuming and labor intensive. Both methods allow simultaneously the rapid screening of different fragments with variable sizes in a single lane (e.g., products derived by multiplex PCR or after restriction digest of large PCR products), making these techniques particularly useful when large regions of DNA have to be screened in many patients. The main disadvantages of both tests is the fact that not all mutations are being detected. In addition, the size of fragments to be analyzed is limited, especially in SSCP. Close to 100% of mutations may be detectable by combining both techniques. However, no systematic evaluations of this assumption is known to us.

It is now well established that denaturing or temperature gradient gel electrophoresis detects close to 100% of point mutations. Comparative studies prove that TGGE *[9]* or DGGE *[54]* detects a higher proportion of point mutations than SSCP. Thus, if the detection of close to 100% of point mutations is intended, DGGE or TGGE should be applied. However, probably because of the relative ease of setup of SSCP, to date, published studies involving this method outweigh DGGE and TGGE roughly fourfold. A major disadvantage of TGGE or DGGE is that running conditions must be defined for each PCR product before analysis. Thus, compared with HET and SSCP, screening of large genes with many exons will be very labor intensive and time consuming, making these methods inefficient for some applications. At present, all of the electrophoretic methods are evaluated by visual inspection, which makes standardization difficult. Capillary electrophoresis may allow a more objective measurement in the future. In the chemical cleavage method, all point mutations are detected and large fragments can be screened. Because a number of analytical steps are required and toxic chemicals are applied, reports on the application of CCM are not as abundant when compared with the above methods. Mismatch cleavage by resolvases holds promise for the future, since standardization and automation should be achieved with relative ease. A major advantage of the mismatch cleavage method is that the size of the cleaved product roughly indicates the localization of the mutation. However, more experience is needed with these methods. The application of mismatch binding proteins has a great appeal for a simple screening test. However, discrimination of hetero- and homodu-

Table 1. Methods for detection of unknown point mutations.

Method	Maximum fragment size (kb)	Detectable mutations	Detection limit (minimal ratio of mutant to wild-type cells)	Detection methods	Potential for standardization and automation[a]	Position of mutation defined
DGGE, TGGE	1	Close to 100%	?	Strand labeling; ethidium bromide or silver stains	Limited	No
SSCP	0.2	80–90%	0.1	Strand labeling; silver stain	Limited	No
HET	1	80%	0.2	Strand labeling; ethidium bromide or silver stains	Limited	No
CCM	2	100%	0.1	Strand labeling; ethidium bromide or silver stain	Limited	Yes
PTT	Depends on electrophoretic resolution of proteins	Mutations generating stop codons	?	Radioactive labeling of translated proteins; protein stains	Limited	Yes
DNA chips	Depends on array	Close to 100%	?	Fluorescence stains; epifluorescence confocal scanning microscopy	High	Yes

RNAse A cleavage is not suited for screening purposes because the rate of detectable mutations is too low; the application of enzyme mismatch cleavage or mismatch binding proteins requires additional experience.

[a] Standardization and automation may be improved by capillary electrophoresis.

plices is not satisfactory at present. The application of PTTs will be restricted to the screening of genes in which translation-terminating mutations are abundant.

For all of the screening methods discussed so far, sequencing is advisable not only for the confirmation of results but also to assess the pathological significance of a specific mutation. Moreover, without sequence confirmation, unknown polymorphisms not associated with disease or technical artifacts may be misinterpreted as mutations, leading to false interpretations. Compared with the sequencing methods used initially, improvements have been achieved by the introduction of semiautomated high-throughput sequencing systems. However, as fully automated sequencing systems are not available at the moment, semiautomated sequencing is still labor intensive, costs are high, and assay performance as well as interpretation of results needs specialized personnel.

Many of the problems may be solved in the future by the introduction of DNA chip technology, which makes possible the combined detection and identification of mutations. However, for many applications, appropriate chips may not be available within the next years. Thus, screening methods for point mutations and small deletions most probably will keep their place in the diagnostic laboratory for a reasonable amount of time.

A major caveat is the lack of methods that are suited for the screening of mutant alleles at low abundance, compared with the wild-type alleles. Such methods are urgently needed for promising applications, such as in tumor diagnosis for the detection of mutant oncogenes in feces and secretions.

Detection of Known Mutations

In one set of methods, mutations are analyzed after the target sequence has been amplified by PCR. Base substitutions are detected by restriction digest, allele-specific hybridization, or by ligation or nonligation of adjacent probes. In a second set of methods, PCR is part of the detection system. The methods rely on the selective extension of primers or on the selective amplification of mutant alleles after restriction digest of wild-type alleles. Only the latter methods allows the sensitive detection of mutant alleles in great excess of wild-type alleles.

NATURALLY OCCURRING OR PRIMER-MEDIATED RESTRICTION FRAGMENT ANALYSIS

Principle. Restriction enzyme recognition sites in DNA, differing because of allelic variation or altered by mutations, are cleaved specifically by restriction endonucleases only when the perfect restriction recognition sequence is present. Commonly, fragments of various sizes are analyzed by gel electrophoresis (restriction fragment length polymorphism, RFLP). In case restriction sites are not affected by mutations, artificial restriction sites can be

introduced into the target DNA by application of mismatched primers for PCR [55, 56].

Modifications. The "mutant-enriched PCR" is the most important modification of this technique. In the original report, artificial, primer-mediated restriction sites were introduced in wild-type DNA by application of mismatched primers localized in the direct vicinity of possible sites of mutations. As a result of base substitutions, uncleavable restriction sites are generated from mutant, but not from wild-type, alleles. Subsequent to the first round of amplification, wild-type DNA is eliminated by restriction digest. In the second round of amplification applying the same primers or heminested primers, ideally PCR products representing mutant alleles only are amplified [57, 58]. Mutant-enriched PCR would also be feasible with natural restriction sites if present.

The transfer of restriction digests from microtiter plates to horizontal gels is greatly facilitated by the so-called "microtiter array diagonal gel electrophoresis" (MADGE) [59, 60].

Detectable mutations. All types of mutations are detectable, for which differences in naturally occurring or primer-generated restriction recognition sequences are present in distinct alleles or wild-type and mutant DNA, respectively.

Limit of detection. By simple RFLP analysis, one mutant cell may be detectable in 50 to 100 nonmutant cells [57]. The detection limit can be lowered significantly by application of the "mutant-enriched PCR" [57]. Repeated restriction digestion and PCR enriched for mutant alleles reportedly allows the detection of one mutant *ras* allele in 10^6 normal alleles [61]. According to our experience, mutant-enriched PCR is well suited to screen for mutant K-*ras* alleles in the stools of patients with colorectal cancer [62].

Methods of detection. Generally, fragments are analyzed by electrophoresis in agarose gels and ethidium bromide staining. Other methods of detection (e.g., hybridization, immobilization of labeled fragments) are less common.

Performance and quality assessment. For RFLP analysis, a specificity of 100% is achieved when appropriate restriction enzymes are used. As quality controls, different allelic variants or wild-type and mutant DNA must be included in each analysis. Recognition sequences may be destroyed by errors of the *Taq* polymerase. In general, errors due to misincorporations will become detectable only when high numbers of PCR cycles and (or) sensitive detection methods are used. In the mutant-enriched PCR, false-positive results will be obtained when a critical number of cycles is exceeded in the second PCR subsequent to the restriction [57]. The method has to be adjusted to conditions such that no false-positive results are obtained when variable amounts and different proportions of wild-type and mutant DNA are analyzed. Questionable results may be confirmed by repetition of experiments and subsequent sequencing of PCR products.

ASO

Principle. Mispairing of a single nucleotide within a hybrid of 20 bp results in a decrease of the T_m of ~5–7.5 °C. This difference in melting temperatures is adequate for the specific detection of single nucleotide exchanges in DNA by oligonucleotides. Cross-hybridization to irrelevant DNA sequences is avoided by oligonucleotides with a minimum size of 16 to 20 bp. Hybridization with larger oligonucleotides does not increase sensitivity, because differences in T_m due to mispairing of nucleotides decreases with increasing fragment length. Generally, one of the reaction partners is immobilized to a solid support.

Modifications. Originally, electrophoretically separated restriction fragments were immobilized on membranes and discriminated by oligonucleotide hybridization [63, 64]. In more recent applications, the target DNA, generally obtained by PCR, was immobilized to membranes without gel electrophoresis (dot blot). The original dot-blot method is laborious when different allelic fragments (e.g., HLA locus) or various mutated fragments (e.g., *CFTR* gene) are used to probe immobilized target fragments. Methodological improvement has been achieved by the reverse dot-blot technique where different oligonucleotides are immobilized to the same membrane, allowing the detection of different polymorphisms or mutations in a single reaction [65]. Further improvement has been achieved by microtiter formats. For the detection of low amounts of K-*ras* mutated cells in a large background of nonmutated cells (e.g., for the detection of tumor cells in stools of patients with colorectal or pancreatic carcinoma), DNA was amplified and cloned. Subsequently, wild-type and mutant clones were discriminated by ASO [66]. In an electrophoretic variant, hybrids of target sequences and labeled oligonucleotides were submitted to electrophoresis in a horizontal 20% polyacrylamide gel at a temperature gradient increasing with time. At the appropriate melting temperature, the oligonucleotide was released. Thus, the freed rather than the bound oligonucleotide is displayed. This technique has been designated "profiling of oligonucleotide dissociation gel electrophoresis" (PODGE) [67]. Hybridization reactions can also be performed in solution. For example, biotinylated primers were used to amplify a fragment of the α_1-antitrypsin gene containing a potential Z-mutation. Hybridization was performed in solution with Eu-labeled matching or mismatch primers. After immobilization on streptavidin-coated wells, mutations were detected by washing at appropriate stringency [68].

ASO hybridization is the principle on which the design of DNA chips is based. DNA chips may be available in the near future, which make possible screening for a wide range of mutations and polymorphisms once these have

been defined. In a recent variation of the chip technology, a contiguous stacking hybridization technique was applied for the detection of β-thalassemia mutations *[69]*.

Detection limit. In reconstruction experiments, one cell with a mutated *ras* gene was detectable in 10 cells with wild-type alleles by the dot-blot technique *[70]*. A large increase in sensitivity was achieved by prior cloning of PCR fragments and screening of individual clones with probes complementary to different mutations of the K-*ras* gene. In this setting, errors of the *Taq* polymerase may give rise to false-positive results. Consequently, a cutoff of positive clones must be established *[66]*.

Detection methods. Originally, ^{32}P-labeled probes were used for detection. For nonradioactive detection in direct or reverse dot blots, avidin–peroxidase conjugate can be applied in combination with biotinylated oligonucleotides or probes *[65]*. Detection of bound probes by time-resolved fluorometry has been mentioned *[68]*.

Performance and quality assessment. The specificity of ASO depends on accurate control of the hybridization conditions. Because they depend on base sequence and particular base substitutions, hybridization conditions must be defined precisely for each application. In solid-phase applications, the effect of base composition on the melting temperature can be minimized by the addition of tetramethylammonium chloride during hybridization. In the direct dot blot, signal intensity is influenced by the affinity of the DNA to the membrane. Similarly, in the reverse dot blot, different signal intensities may be obtained when multiple oligonucleotides are immobilized to a different extent. To reach comparable signal intensities, the concentrations of immobilized oligonucleotides must be adjusted *[65]*. Difficulties of interpretation may occur when weak signals are obtained. For each analysis, matching and mismatching controls have to be included for each allele or each mutation, respectively. Samples and controls should be analyzed on the same membrane when different samples are investigated in parallel. Standardization is difficult when signal intensities are evaluated by visual inspection. The use of microtiter plates, other surfaces, or tubes in conjunction with devices to measure signal intensities of bound hybrids appears to be better suited, both for automation and standardization. In this context, the DNA chip technology represents a major breakthrough. Many of the problems associated with conventional solid-phase applications are avoided by the PODGE variant *[67]*.

ALLELE-SPECIFIC AMPLIFICATION (ASA)

Principle. PCR is performed in two parallel reactions. In the first reaction, the 5′ primer is complementary to the wild-type sequence; in the second reaction, the 5′ primer is complementary to the mutant or polymorphic sequence. Assuming that elongation occurs only when primer and target sequence match completely, only one allele of either mutant or wild-type DNA is amplified. The method was developed independently by different groups. Two different approaches have been described in parallel. The first approach is based on the lack of primer elongation due to a mismatch at the far 3′-end of the primer. These methods have been named "amplification refractory mutation system" (ARMS) *[71]*, "allele-specific PCR" (ASPCR) *[72]*, "PCR amplification of specific alleles" (PASA) *[73]*, or ASA *[74]*. In the second approach, the mismatch is located within the primer, preventing primer annealing when mispairing occurs. Methods based on this principle were called "competitive oligonucleotide priming" (COP) *[75]* or "color complementation assay" (CCA) *[76]*.

Modifications. Assuming a homozygous situation, lack of amplification will occur in one of the reactions when PCR is performed with different pairs of 5′ primers, one complementary and the other not complementary to the alleles. Internal controls must be included to exclude false-negative results (e.g., for heterozygosity). By multiplex PCR, developed, e.g., for the diagnosis of cystic fibrosis, a positive signal is obtained in each reaction, circumventing this problem and allowing the simultaneous detection of different alleles *[77]*. A further disadvantage of the original protocols is the performance of two different reactions in parallel. Heterozygous or homozygous status may be discriminated in a single reaction when different alleles are amplified by primers labeled with different fluorochromes *[76, 78]*. In the ASA by tetra-primed PCR, different alleles can be distinguished in a single PCR, by using two annealing temperatures and four primers *[79]*.

Both pairs of primers elongating either one or the other allele can be applied in one reaction when the methodological variant "PCR amplification of multiple specific alleles (PAMSA)" is performed. One of the allele-specific primers carries an additional stretch of noncomplementary nucleotides at the 5′-end. Thus, amplification products of both alleles can be discriminated by differences in size *[80, 81]*. A comparable method has been described as "double ARMS" *[82]*.

Detectable mutations. Depending on assay conditions and mismatch, false extension of 3′-ends of primers may occur. Kwok et al. *[83]* reported that yield of PCR products decreased by 100-fold for A:G, G:A, or C:C mispairing and by 20-fold for A:A mispairing. Elongation of primers occurred in all other types of mispairing. However, elongation of mismatched bases can be avoided when appropriate primers and reaction conditions are applied. Specificity of primer extension may be improved by appropriate adjustment of experimental conditions *[84, 85]*. Specificity of the reaction is influenced by the concentration of magnesium, primers, deoxyribonucleotides, target DNA, and *Taq* DNA polymerase. Addition of

formamide may reduce unspecific reactions. Under optimized conditions all types of mismatches can be reproducibly detected by ASPCR at comparable concentrations of different alleles or wild-type and mutant DNA *[84]*. Furthermore, a more reliable inhibition of elongation is achieved by introduction of additional mismatches 5′ of the 3′ end of the primer *[77]*. The specificity of the method is strongly influenced by the ratio of mutant to wild-type DNA.

Limit of detection. Identification of a homozygous or heterozygous state is the main application of the ASA. This method is reliable and flexible for the analysis of homozygous or heterozygous states. Specificity is more critical when the ratio of mutant to wild-type alleles is low and the actual fraction of mutant alleles is unknown. The detection of few tumor cells carrying mutations of the K-*ras* gene in the presence of a large number of normal cells has been reported by several authors *[86–88]*.

Methods of detection. For most of the methods outlined above, detection is performed by gel electrophoresis. Similar to other applications, a main advantage of electrophoretic detection systems is the possibility to control the appropriate size of fragments. By application of fluorochrome-labeled primers, electrophoretic separation is not necessary when primers in excess are removed before detection *[76]*.

Performance and quality assessment The possibility of false-positive or -negative results is the major limitation of ASA. False-positive results may be due to contamination or imperfect extension. Guidelines regarding the avoidance of contaminations should be followed strictly. To exclude false results, reaction conditions must be standardized and the concentration of target DNA must be defined and controlled precisely. Target alleles should be included as controls to exclude false-positive or false-negative results. Primers complementary to alleles with and without mismatches should both be used. Automation of the method by the use of solid supports and nonradioactive detection systems is conceivable.

SINGLE NUCLEOTIDE PRIMER EXTENSION

Principle. The principle is similar to that of ASA. The method is based on the extension of the 3′-end of a primer by a single labeled nucleotide. Extension occurs only when the labeled nucleotide is complementary to the nucleotide of the target DNA adjacent to the 3′-end of the primer *[89, 90]*. On the basis of comparable fidelities, either T7 or *Taq* DNA polymerases can be applied. Because of the high error rate, the Klenow fragment of *E. coli* DNA polymerase is not suited *[91]*. The method is also known as "minisequencing."

Modifications. In one of the first reports on this method, two different reactions were performed with labeled nucleotides either complementary to one allele or to the other allele *[90]*. In the second approach, specific nucleotides were applied, leading to differences in the electrophoretic mobility of the fragments *[89]*. In addition, similar approaches, with modifications mainly in the labeling strategies, have been reported by several authors *[91, 92]*. One modification is based on the method of dideoxy sequencing. The 3′-end of the primer is located upstream of the mutant nucleotide(s). Use of dideoxy nucleotides complementary to the mutant nucleotide primer extension will lead to a termination earlier in mutant alleles than in wild-type alleles *[93]*.

Specificity. Reliable discrimination between different alleles will be obtained when the reaction is performed under appropriate conditions. Application of comparable amounts of different DNA to be analyzed is required.

Detectable mutations. All possible types of nucleotide exchanges can be detected by the single nucleotide primer extension method.

Detection limit. No systematic analyses on the performance of the technique at different ratios of wild-type to mutant DNA are known to us.

Detection methods. Nucleotides are either labeled by ^{32}P *[90]*, ^{3}H, or by digoxigenin *[91]*. In general, products of primer extension are analyzed by electrophoretic separation *[90, 92, 94]*. In one of the original approaches, nucleotides were applied, modifying the electrophoretic mobility of the fragments *[89]*. The method has been performed as a solid-phase technique, making automation possible *[91, 95]*.

Performance and quality assessment. Because signals can be quantified without electrophoresis, the method is well suitable for automation. A nonradioactive automated solid-phase assay has been described *[91, 95]*.

Diagnosis is based on the comparison of the results with appropriate controls (homozygous, heterozygous, mutant vs wild-type). For reliable discrimination, minimal variation of positive signals and background is essential, making standardized amounts of DNA mandatory.

OLIGONUCLEOTIDE LIGATION ASSAY (OLA)

Principle. Two primers are hybridized to complementary stretches of DNA at sites of possible polymorphisms or mutations; primers are created such that the 3′-end of the first primer is located immediately adjacent to the 5′-end of the second primer. Assuming that the 3′-end of the first primer matches perfectly with the target DNA, both primers can be ligated by DNA ligases (e.g., T4 DNA ligase). No ligation will be obtained when a mismatch occurs at the 3′-end of the first primer *[96, 97]*.

Modifications. In the original approach, ligated and nonligated primers were discriminated by dot blot on the basis of differences in hybridization conditions *[96]*. A second approach involved labeled primers where the first primer was biotinylated at the 5′-end and the second primer was ^{32}P- or fluorochrome-labeled at the 3′-end. Differentiation of fragment size by electrophoresis is feasible.

Detectable mutations. All possible combinations of base pairings between the 3′-end of the 5′ primer and the target DNA have been investigated. Under appropriate conditions, ligation will take place only when the 3′-end of the primer matches perfectly with the target sequences *[97]*.

Limit of detection. Studies on the specificity and sensitivity of the method at variable ratios of mutant to wild-type DNA are not known to us.

Performance and quality assessment. Ligation of complementary bases depends mainly on the concentration of salts and the proportions of concentrations between ligase and DNA. False-negative results will be obtained when high salt concentrations or low concentrations of enzyme are applied *[97]*. A low variability of positive signals and background signals is essential for reliable discrimination. Standardization of DNA extraction is essential. Positive and negative controls should be included in each assay.

An automated version of OLA has been described *[98]*. For detection, the 5′-end of one primer was labeled with biotin and the 3′-end of the other primer was labeled with digoxigenin. After ligation and binding to streptavidin immobilized on the surface of a microtiter plate, digoxigenin was detected by an anti-digoxigenin antibody coupled to alkaline phosphatase catalyzing a substrate reaction.

Detection of Known Mutations: Summary and Conclusions

Methods for the detection of known point mutations and small deletions or insertions are summarized in Table 2.

Any one of the above methods is suited for the analysis of allelic differences in hereditary disease. Non-gel-based detection systems have been developed for most of the assays described, making these methods favorable for application in routine laboratories. For each technique, reaction conditions must be standardized and appropriate internal controls must be included. One must keep in mind that misleading results may be obtained because of unknown polymorphisms within the target region affecting, e.g., restriction enzyme recognition sequences or hybridization of probes and binding of primers.

In cases in which a large number of different mutations or polymorphisms are to be detected, the DNA chip technology most probably will be the method of choice in the near future. However, with a restricted set of mutations such as the factor V gene mutation in activated protein C resistance, methods that are technically less demanding will keep their place in clinical laboratories.

At present, primer-mediated restriction fragment analysis in conjunction with mutant-enriched PCR appears

Table 2. Methods for detection of known point mutations.

Method	Detectable mutations	Detection limit (minimal ratio of mutant to wild-type cells)	Detection methods	Performance and quality assessment
Restriction fragment analysis	All base exchanges that destroy recognition sequences for restriction enzymes	Without enrichment: 0.05 Mutant enriched: 10^{-4}–10^{-5}	Electrophoresis; ethidium bromide staining of fragments; solid-phase formats possible	Robust technique; *Taq* polymerase errors may create false positives in mutant-enriched PCR
ASO	All base exchanges	Without cloning: 0.1 Screening of cloned PCR products: 10^{-4}–10^{-5}	Direct and reverse dot blot, other solid-phase formats (DNA chips); electrophoresis; radioactive labels; nonradioactive labels (e.g., biotin, fluorochromes)	Depends on particular technical modifications; *Taq* polymerase may create false positives in ASO with cloned fragments
ASA	All base exchanges	10^{-4}–10^{-5}	Electrophoresis; solution phase; radioactive labels, nonradioactive labels (e.g., fluorochromes)	Ratio of mutant to wild-type alleles must be in a defined range to avoid false results; automation feasible
Single nucleotide primer extension	All base exchanges	?	Solid-phase assays; radioactive labels; nonradioactive labels (e.g., digoxigenin)	Ratio of mutant to wild-type alleles must be in a defined range to avoid false results; automation feasible
OLA	All base exchanges	?	Electrophoresis; capture of biotinylated oligonucleotides in solid-phase formats; radioactive labels; nonradioactive labels (e.g., fluorochromes)	Salt and enzyme concentrations critical; automation feasible

the technique best suited for the amplification of low-abundance mutated alleles in great excess of nonmutated alleles. In comparison with ASO hybridization after cloning of PCR fragments, primer-mediated restriction fragment analysis is technically simpler and sufficiently sensitive. So far, promising applications of the technique are the detection of K-*ras* mutations in stools *[62]* and bronchoalveolar lavage *[99]*.

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